THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 19, pp. 13661–13666, May 9, 2014

The Human Enzyme That Converts Dietary Provitamin A Carotenoids to Vitamin A Is a Dioxygenase*

Received for publication, February 12, 2014, and in revised form, March 19, 2014 Published, JBC Papers in Press, March 25, 2014, DOI 10.1074/jbc.M114.557710

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Background: The human enzyme β-carotene 15–15′-oxygenase (BCO1) has been thought to be a monooxygenase.
Results: Incubation of BCO1 and β-carotene in $H_2{}^{18}O$-$^{16}O_2$ or $H_2{}^{16}O$-$^{18}O_2$ medium yields two retinals both of which contain oxygen atoms originating solely from O_2 gas.
Conclusion: BCO1 is a dioxygenase.
Significance: It is important to clearly establish the reaction mechanism of an enzyme, especially when the name reflects the mechanism.

β*-Carotene 15–15′-oxygenase (BCO1) catalyzes the oxidative cleavage of dietary provitamin A carotenoids to retinal (vitamin A aldehyde). Aldehydes readily exchange their carbonyl oxygen with water, making oxygen labeling experiments challenging. BCO1 has been thought to be a monooxygenase, incorporating oxygen from O_2 and H_2O into its cleavage products. This was based on a study that used conditions that favored oxygen exchange with water. We incubated purified recombinant human BCO1 and *β*-carotene in either $^{16}O_2$-$H_2{}^{18}O$ or $^{18}O_2$-$H_2{}^{16}O$ medium for 15 min at 37 °C, and the relative amounts of ^{18}O-retinal and ^{16}O-retinal were measured by liquid chromatography-tandem mass spectrometry. At least 79% of the retinal produced by the reaction has the same oxygen isotope as the O_2 gas used. Together with the data from ^{18}O-retinal-$H_2{}^{16}O$ and ^{16}O-retinal-$H_2{}^{18}O$ incubations to account for nonenzymatic oxygen exchange, our results show that BCO1 incorporates only oxygen from O_2 into retinal. Thus, BCO1 is a dioxygenase.

Vitamin A deficiency is the most common vitamin deficiency in the world and affects an estimated 190 million preschool-age children and 19.1 million pregnant women worldwide.[2] In areas of endemic vitamin A deficiency, people obtain vitamin A almost exclusively as provitamin A carotenoids found in foods of plant origin (2). Provitamin A carotenoids are enzymatically converted to retinal (vitamin A aldehyde) (Fig. 1*A*) by the enzyme β-carotene 15–15′-oxygenase (BCO1)[3] (3). Hence, understanding the mechanism and regulation of this enzyme is important.

The reaction mechanism, and consequently, the nomenclature of BCO1 and other carotenoid cleavage oxygenases (CCOs) have been controversial (4–6). The first report of a CCO was made in 1965 by Goodman and Huang (7), who showed that β-carotene was converted to retinal by cell-free rat intestinal homogenates in the presence of O_2. The following year, the same group then showed using ^3H labels that the hydrogens of the 15–15′ double bond of β-carotene (the site of oxidative cleavage) are retained during the enzymatic oxidation reaction, and they proposed that the reaction most likely has a dioxygenase mechanism (8). However, the label "dioxygenase" should only be used when oxygen labeling experiments have clearly established that only oxygen from O_2 is incorporated by the enzyme into its oxidative cleavage products. BCO1 was given the Enzyme Commission (EC) number 1.13.11.21 in 1972, designating a dioxygenase (9), 29 years before the first report of an oxygen labeling experiment. A monooxygenase mechanism was proposed for BCO1 in 2001 (10). In that study, α-carotene, purified chicken BCO1 and horse liver alcohol dehydrogenase were incubated in an 85% $^{17}O_2$-95% $H_2{}^{18}O$ environment. Horse liver alcohol dehydrogenase was used to form retinols from the aldehydes, which readily exchange their carbonyl oxygen with water (11). The resulting products (retinol and α-retinol) were purified by high performance liquid chromatography and silylated. Using gas chromatography-mass spectrometry (GC-MS), the authors found virtually equal enrichment of ^{17}O and ^{18}O in both silylated retinols, suggesting a monooxygenase mechanism (Fig. 1*B*). However, it is possible that the long reaction time (7.5 h) and extensive processing favored oxygen exchange between the initial aldehyde products and the aqueous medium. Also, the horse liver alcohol dehydrogenase reaction is reversible, and the enzyme displays dismutase activity (interconverting the aldehyde into alcohol and carboxylic acid) (6, 12). This means that the aldehydes were never completely eliminated during the 7.5-h incubation, and a significant amount of oxygen exchange with water may have occurred. Despite the inconclusiveness of this study, the

* This work was supported, in whole or in part, by National Institutes of Health Grant R01-HL49879 (NHLBI and the National Institutes of Health Office of Dietary Supplements). This work was also supported by grants from The Ohio State University Food Innovation Center and the Ohio Agricultural Research Development Center. The mass spectrometry experiments were done at the Nutrient and Phytochemical Analytic Shared Resource of the Ohio State University Comprehensive Cancer Center.
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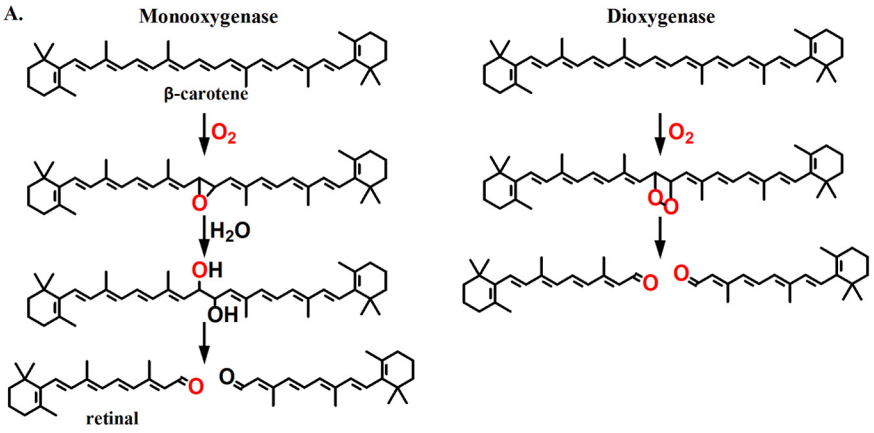
[2] World Health Organization, unpublished data.
[3] The abbreviations used are: BCO1, β-carotene 15–15′-oxygenase; CCO, carotenoid cleavage oxygenase; MS/MS, tandem mass spectrometry; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.



B. Theoretical results

% ^{18}O-retinal			
$^{16}O_2$-$H_2{}^{18}O$		$^{18}O_2$-$H_2{}^{16}O$	
Monooxygenase	Dioxygenase	Monooxygenase	Dioxygenase
50	0	50	100

C. Experimental results

% ^{18}O-retinal	
$^{16}O_2$-$H_2{}^{18}O$	$^{18}O_2$-$H_2{}^{16}O$
BCO1 + β-carotene	BCO1 + β-carotene
3, 6, 10	79, 85
BCO1 + ^{16}O-retinal (>99% atom)	BCO1 + ^{18}O-retinal (91% atom)*
5, 7, 13	67, 84

* BCO1 + ^{18}O-retinal incubated in $^{16}O_2$-$H_2{}^{16}O$.

FIGURE 1. **Human BCO1 is a dioxygenase.** *A*, putative reaction mechanisms of BCO1. A monooxygenase incorporates an oxygen atom from O_2 in one retinal molecule and an oxygen atom from water into the other (10). A dioxygenase incorporates only atoms from O_2 into the cleavage products (8). *B*, theoretical percentages of ^{18}O-retinal that will be obtained for oxygen labeling experiments with BCO1 as a monooxygenase and as a dioxygenase. *C*, summary of results of oxygen labeling experiments with purified recombinant BCO1. The *numbers* separated by *commas* are the percentage ^{18}O enrichment of the retinal product from individual experiments done on different days. Due to limited supply of $^{18}O_2$, only two BCO1-β-carotene reactions were done in $^{18}O_2$-$H_2{}^{16}O$. Retinal obtained from the BCO1-β-carotene reaction contains predominantly the same oxygen isotope as O_2. Control incubations of active BCO1 with ^{18}O-retinal in $H_2{}^{16}O$ and ^{16}O-retinal in $H_2{}^{18}O$ account for the oxygen exchange that occurred in the corresponding BCO1-β-carotene reactions. Thus, BCO1 incorporates solely oxygen from O_2 during the oxidative cleavage of β-carotene and is therefore a dioxygenase. The isotopic purity of the ^{16}O-retinal standard is based on natural abundance of ^{16}O (17) and verified by LC-MS/MS. Isotopic purity of synthesized ^{18}O-retinal was measured by LC-MS/MS.

enzyme EC number was changed to 1.14.99.36, classifying it as a monooxygenase (9), and subsequent literature has referred to the animal orthologues of the enzyme as β-carotene 15–15′-monooxygenase (BCMO1). Indeed, the National Center for Biotechnology Information named the gene *BCMO1*.[4]

To elucidate the reaction mechanism of human BCO1, we conducted multiple oxygen labeling experiments with minimal reaction and processing times to minimize oxygen exchange between retinal and water. Our results demonstrate that BCO1 is not a monooxygenase, but a dioxygenase.

EXPERIMENTAL PROCEDURES

Chemicals—β-Carotene (≥97%), all-*trans*-retinal (≥98%), $H_2{}^{18}O$ (97% atom) and $^{18}O_2$ gas (99% atom) and Dowex 50W-X4 were purchased from Sigma-Aldrich.

Synthesis of ^{18}O-retinal—All-*trans*-retinal (20 nmol), $H_2{}^{18}O$ (200 equivalents), 2 ml of acetonitrile, and 60 mg of Dowex 50W-X4 (hydrogen form) were stirred at room temperature in a closed vial protected from light for 1.5 h. This is based on the method of Kawanishi *et al.* (15). The solids were removed by decantation, and the retinoids were then extracted with 3 × 2-ml hexanes. The hexane extracts were combined and stored at −80 °C. The final product is 91% ^{18}O-retinal as measured by LC-MS/MS (below).

Freeze-drying of Purified Recombinant Human BCO1—Purified recombinant human BCO1 was prepared according to our previously published method (16). The purified enzyme preparation catalyzed the oxidative cleavage of β-carotene with a V_{max} = 197.2 nmol of retinal/mg of BCO1 × h, K_m = 17.2 μm and catalytic efficiency k_{cat}/K_m = 6098 M^{-1} min^{-1}. Ten μg of purified recombinant human BCO1 and 40 μl of 5× reaction buffer (500 mM Tricine-KOH, pH 8.0, at 37 °C, 2.5 mM dithiothreitol, 20 mM sodium cholate, 75 mM nicotinamide) (16) were combined in a 10-ml amber headspace vial, and the vial was capped and flash-frozen in liquid nitrogen. The headspace vials were stored in dry ice for 30 min during transport to the freeze-dryer. The caps of the headspace vials were then fitted with individual syringe needles for venting, and the vials were placed in the jar of the manifold freeze dryer (Labconco). Freeze-drying was done for 16 h at 0.14 mBar. The syringe needles were removed, and the headspace vials were stored at −80 °C until use. Each vial of freeze-dried enzyme produces approximately 60 pmol of retinal from 4 nmol of β-carotene with the *in vitro* BCO1 activity assay system described in the following section.

In Vitro BCO1 Activity Assay in $^{16}O_2$-$H_2{}^{18}O$—The *in vitro* enzyme assay using purified recombinant human BCO1 was

[4] National Center for Biotechnology Information, unpublished data.

based on our previously published method (16). The freeze-dried enzyme-reaction buffer mixture in the headspace vial (described in previous section) was dissolved in $H_2{}^{18}O$ to a final volume of 160 μl and placed in a 37 °C shaking water bath. The reaction was initiated by adding 40 μl of β-carotene substrate solution (containing 4 nmol of β-carotene, 0.3 μl of Tween 40, and 20 nmol of α-tocopherol) prepared in $H_2{}^{18}O$. The reaction was allowed to proceed in the water bath with gentle shaking and the vial exposed to air (which contains oxygen as 99.8% $^{16}O_2$ (17)) for 15 min. The quenching with 37% formaldehyde in the original method had to be omitted because the latter contains $H_2{}^{16}O$. Instead, the reactions were quenched with 300 μl of acetonitrile, and the lipophilic compounds were extracted with 3 × 1 ml of hexanes under red lights. The combined hexane extracts were dried under N_2, redissolved in 100 μl of 3:1 (v/v) acetonitrile-$H_2{}^{18}O$, filtered through a 0.22-μm syringe-driven filter, and injected into the HPLC. The whole process from the start of the reaction to the elution of the retinal peak in the HPLC takes approximately 50–60 min.

In Vitro BCO1 Activity Assay in $^{18}O_2$-$H_2{}^{16}O$—The enzyme-reaction buffer solution (10 μg of purified recombinant human BCO1, 40 μl of 5× reaction buffer, and water to a total volume of 160 μl) was placed in a headspace vial and degassed by exposure to water aspirator vacuum for 2 min. The headspace vial was purged with nitrogen gas, then connected to the $^{18}O_2$ gas cylinder and placed in a 37 °C water bath. Forty μl of β-carotene substrate solution, prepared in degassed water ($H_2{}^{16}O$), was then injected into the vial using a syringe. The reaction was allowed to proceed in the water bath with gentle shaking for 15 min. The reaction was quenched by injecting 300 μl of acetonitrile into the vial before the $^{18}O_2$ gas flow was turned off. The reaction mixture was then extracted and processed as in the previous section, except that the extract residue was redissolved in acetonitrile-$H_2{}^{16}O$.

Control Experiments—To account for the oxygen exchange between water and retinal, we incubated 60 pmol of all-*trans*-retinal (^{16}O-retinal) in the reaction mixture prepared in $H_2{}^{18}O$ containing active BCO1, as described above for "*In vitro* BCO1 activity assay in $^{16}O_2$-$H_2{}^{18}O$." We also performed an analogous incubation using our synthesized ^{18}O-retinal in $H_2{}^{16}O$.

UHPLC-MS/MS Method—The reaction mixture was separated by an Agilent 1290 UHPLC system (Agilent Technologies) using a Zorbax Extend 2.1 × 50-mm, 1.8-μm C18 column (Agilent Technologies). The flow rate was 0.8 ml/min, and the column temperature was 40 °C. The composition of solvents was as follows: A = 0.1% formic acid in water; B = 0.1% formic acid in acetonitrile. A linear eluting gradient was applied as follows: isocratic 60% B for 0.5 min, gradient from 60 to 78% B over 3 min, gradient from 78 to 100% B over 1.5 min, isocratic 100% B for 2 min, and re-equilibration to 60% B over 2 min.

The HPLC was interfaced with an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies) using an electrospray ionization source operating in positive ion mode. The MS instrumental parameters included: sheath gas temperature, 400 °C; flow rate, 12 liters/min; drying gas temperature, 150 °C; flow rate, 15 liters/min; 3-Hz MS/MS acquisition; 10-Hz MS reference scans; 30 psig nebulizer; Vcap, 2000 V; nozzle voltage, 2000 V; fragmentor, 350 V; ion funnel settings for small molecules. MS/MS transitions were acquired by collision-induced dissociation of all-*trans*-retinal standard (m/z = 285.218) and ^{18}O-retinal (m/z = 287.226) and found to optimize at a collision energy of 7.5 eV. Source and collision-induced dissociation gas was high purity (>98%) nitrogen. Calibration was performed using ESI-L tuning mix (Agilent Technologies G1969-85000), and within-run reference compound was hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine, m/z 922.010 (Agilent Technologies HP-0921).

Quantification of Retinal Oxygen Isotopologues—The fragmentation patterns of ^{18}O-retinal and ^{16}O-retinal are virtually the same (see Fig. 3). The parent retinals were not used for quantification to minimize errors arising from other naturally occurring isobaric species, which constitute approximately 2.2% based on the natural abundance of ^{13}C (17). MS/MS was used to discriminate between the retinal analytes from these isobaric species, which will give different fragmentation patterns.

For quantification of the retinal oxygen isotopologues, the MS/MS fragments m/z = 119.086, 175.150, 105.070, 133.101, 163.101, 195.163, and 231.162 for ^{18}O-retinal and m/z = 119.086, 175.143, 105.070, 133.101, 161.092, 193.159, and 229.158 for ^{16}O-retinal were summed to generate one extracted ion chromatogram for each parent retinal species. These daughter ions were selected because they were the dominant fragment ions. Also, the last three daughter ions listed for ^{18}O-retinal and ^{16}O-retinal differ by 2 atomic mass units, indicating that these fragments bear the oxygen atom.

RESULTS

For the BCO1-β-carotene reaction in $^{16}O_2$-$H_2{}^{18}O$ medium, the retinal product obtained (approximately 60 pmol) after a 15-min reaction was only about 3–10% ^{18}O-retinal (Fig. 1*C*). This range reflects what we obtained from three experiments done on different days. A sample LC-MS chromatogram is shown in Fig. 2*A*. The small relative amount of ^{18}O-retinal we observed in $^{16}O_2$-$H_2{}^{18}O$ medium suggests that BCO1 is a dioxygenase (Fig. 1*B*). If the enzyme is a dioxygenase, then theoretically, it should produce only ^{16}O-retinal, and the ^{18}O-retinal we observed was due to oxygen exchange with water. To verify this, we incubated 60 pmol of ^{16}O-retinal with BCO1 in $H_2{}^{18}O$ under the same conditions. The percentage of ^{18}O-retinal formed was similar (5–13%) to that produced in the reaction of BCO1 and β-carotene (Fig. 1*C*). This confirms that the ^{18}O-retinal we were detecting was coming from the oxygen exchange of retinal with water and not from the enzyme incorporating oxygen from water during the oxidative cleavage reaction.

We then conducted the BCO1-β-carotene reaction in $^{18}O_2$-$H_2{}^{16}O$ medium. Consistent with our previous experiments, the majority of the retinal product obtained contains the same oxygen isotope as that of O_2 (79–85% ^{18}O-retinal). As in the previous section, this range reflects what we obtained from experiments done on different days. A sample LC-MS chromatogram is shown in Fig. 2*B*, and the MS/MS traces for m/z = 285.218 and 287.226 (corresponding to ^{16}O-retinal and ^{18}O-retinal, respectively) are shown in Fig. 3. To verify that the ^{16}O-retinal (15–21%) we observed was due to oxygen exchange with water, we also incubated ^{18}O-retinal (91% ^{18}O-retinal) with BCO1 in

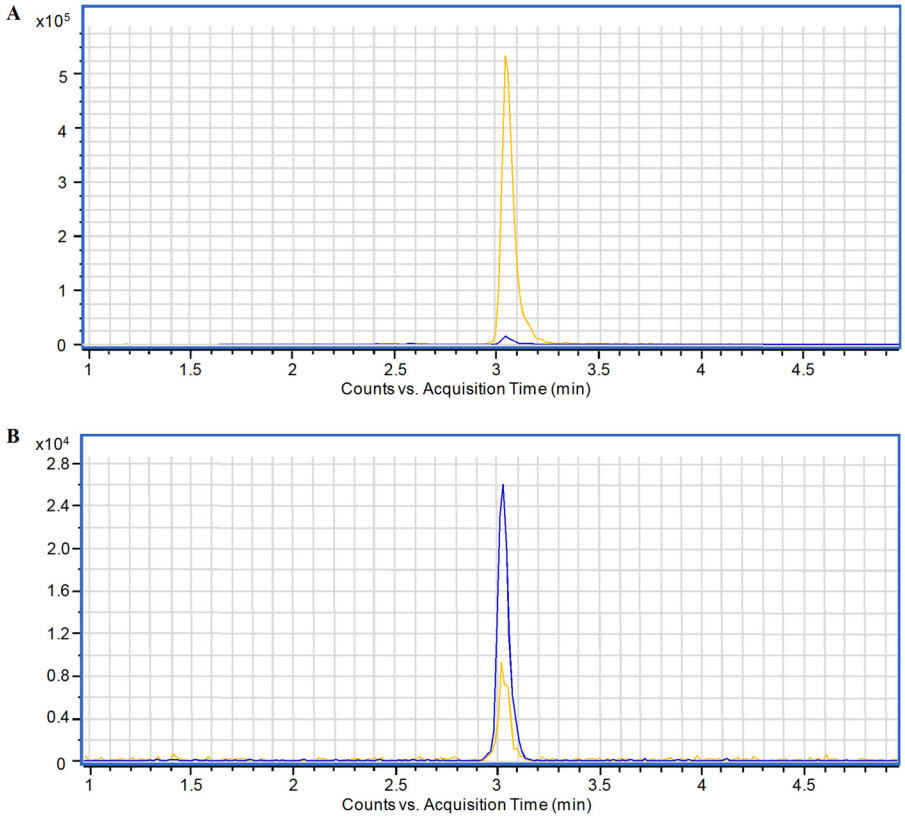


FIGURE 2. **LC-MS chromatograms for the reaction mixture of BCO1 and β-carotene in $^{16}O_2$-$H_2{}^{18}O$ and $^{18}O_2$-$H_2{}^{16}O$.** Purified recombinant human BCO1 (10 μg/200 μl) was incubated with β-carotene (20 μM) for 15 min at 37 °C, and the reaction mixture was analyzed by LC-MS/MS. The LC-MS chromatograms from the reaction mixture in $^{16}O_2$-$H_2{}^{18}O$ (*A*) and $^{18}O_2$-$H_2{}^{16}O$ (*B*) are shown. The *traces* shown are the sum of the fragments from the MS/MS fragmentation of ^{18}O-retinal (*m/z* = 287.226) (*blue trace*), and ^{16}O-retinal (*m/z* = 285.218) (*orange trace*). MS/MS fragments used for quantification are listed under "Experimental Procedures."

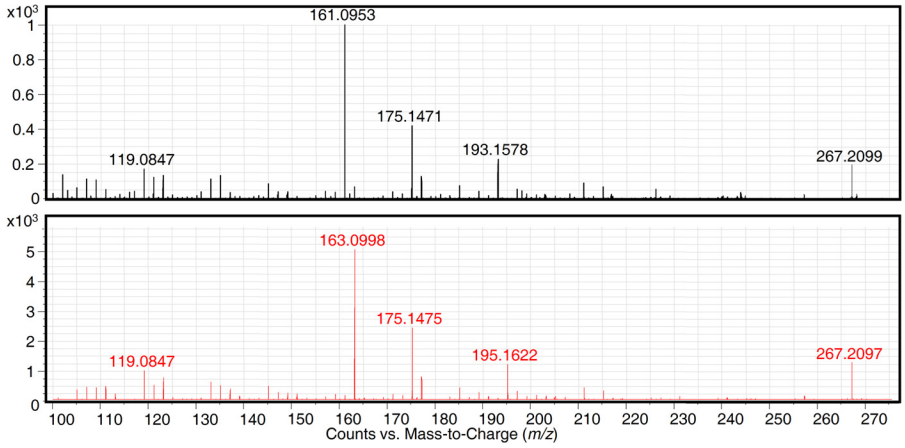


FIGURE 3. **MS/MS traces for the fragmentation of ^{16}O-retinal and ^{18}O-retinal obtained from the reaction of BCO1 and β-carotene in $^{18}O_2$-$H_2{}^{16}O$.** The MS/MS trace for ^{16}O-retinal (*m/z* = 285.218) is shown in the *upper panel*, and that for ^{18}O-retinal (*m/z* = 287.226) is in the *lower panel*.

$H_2{}^{16}O$ under the same conditions. We observed 67–84% ^{18}O-retinal, corresponding to a 7–24% net exchange (Fig. 1*C*). Consistent with the previous section, these values strongly suggest that BCO1 reacts with β-carotene in an $^{18}O_2$-$H_2{}^{16}O$ to form only ^{18}O-retinal, and the small relative amount of ^{16}O-retinal is due to oxygen exchange with water.

We also performed the BCO1-β-carotene incubation in $^{16}O_2$-$H_2{}^{18}O$ for 7.5 h, and the retinal product obtained was 50% ^{18}O-retinal, which verifies that such a long incubation time will indeed lead to a false identification of the enzyme as a monooxygenase.

The $^{16}O_2$-$H_2{}^{18}O$ and $^{18}O_2$-$H_2{}^{16}O$ experiments strongly suggest that BCO1 incorporates only oxygens from O_2 into retinal formed from the oxidative cleavage of β-carotene, and the minor amount of retinal with the same oxygen isotope as water is formed by nonenzymatic oxygen exchange. Thus, BCO1 is a dioxygenase and not a monooxygenase as had been previously thought.

If the parent retinals are used for quantification, the values differ by only 0–6% from the MS/MS calculation (Table 1), and the data still lead to the same conclusion that BCO1 incorporates only oxygen from O_2 into retinal formed from oxidative cleavage of β-carotene.

TABLE 1

Comparison of retinal quantification by MS and MS/MS

The values using the parent ions obtained from LC-MS are in italics and those obtained by using the summation of daughter ions obtained from LC-MS/MS are in regular font. Quantification by either method supports our conclusion that the retinal generated by BCO1 from β-carotene contains the same oxygen isotope as that of O_2.

% ^{18}O-retinal	
$^{16}O_2$-$H_2{}^{18}O$	$^{18}O_2$-$H_2{}^{16}O$
BCO1 + β-carotene 3, 6, 10 *2, 6, 12*	BCO1 + β-carotene 79, 85 *80, 80*
BCO1 + ^{16}O-retinal 5, 7, 13 *11, 6, 15*	BCO1 + ^{18}O-retinal (91%, *89%*) 67, 84 *71, 82*

DISCUSSION

At this point, there is a very limited amount of literature on other CCOs with which to compare our results. Most of the functionally characterized CCOs are from plants, and these enzymes have been called dioxygenases despite the lack of conclusive oxygen labeling experiments (5, 6). This error can be traced back to the lignostilbene dioxygenases. As of 1997, these enzymes were called as such even though no oxygen labeling experiments were carried out (6, 19–23). At best, these studies showed that these enzymes require O_2. This error in naming was propagated into the CCOs in 1997, when the first CCO to be cloned and characterized, maize Viviparous 14, was called a dioxygenase based on its sequence similarity to lignostilbene dioxygenase and not on oxygen labeling experiments (24, 25). Even if the lignostilbene oxygenases were truly established to be dioxygenases back then, a sequence similarity is not necessarily a substitute for oxygen labeling experiments. Interestingly, the first report of an oxygen labeling experiment for a stilbene oxygenase (which was also identified because of sequence similarity to the plant CCOs) showed a monooxygenase reaction mechanism (1).

Of the more than 200 putative CCOs to be found in sequence databases (5), there are only four other oxygen labeling experiments done apart from the aforementioned 2001 BCO1 study. The oxygen labeling experiments on water-stressed leaves of *Xanthium strumarium* in 1984 (13) looked at only one cleavage product, and the *Arabidopsis thaliana* study in 2006 (24) was deemed inconclusive because of the failure to show a consistent labeling pattern for the two cleavage products (Kloer and Schulz give a detailed critique of these two studies (5)). An oxygen labeling experiment was done with *Microcystis* PCC 7806 cells, which generate β-cyclocitral and crocetindial from oxidative cleavage of β-carotene (14). However, the results between the $^{16}O_2$-$H_2{}^{18}O$ and $^{18}O_2$-$H_2{}^{16}O$ incubations were contradictory, and the authors acknowledge that the longer processing time for crocetindial may have favored oxygen exchange. Another oxygen labeling study done with a purified recombinant marine bacterial CCO that also cleaves β-carotene to retinal also shows a dioxygenase mechanism (18), consistent with our results.

Unlike other enzyme names such as "isomerase" or "lyase", the names dioxygenase and monooxygenase both indicate a specific reaction mechanism. Thus, the mechanism should be elucidated first before the name of an oxygenase is assigned. For oxygenases that yield aldehydes, oxygen exchange with water should be minimized and accounted for. BCO1 was called a dioxygenase in 1972 without an oxygen labeling experiment, and a monooxygenase in 2001 despite an inconclusive study. Our results demonstrate that BCO1 is a dioxygenase.

Acknowledgments—We thank Dr. Dennis Bong for letting us use his freeze-dryer for the initial optimization experiments and Jessica Cooperstone, Morgan Cichon, Abby Monnin, and Matthew Teegarden for assistance in freeze-drying at the Schwartz laboratory.

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